Studies on ectomycorrhizae of pine in an electronically air-filtered, air-conditioned, plant-growth room

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An air-filtered, air-conditioned, plant-growth room is described and results of initial studies on ectomycorrhizae of pine in the room are presented. The room was fumigated with methyl bromide and maintained free of airborne spores of ectomycorrhizal fungi for 5 months.

In the room, loblolly pine seedlings in pots of soil infested with forest humus developed ectomycorrhizae, whereas several hundred control loblolly pine seedlings did not form ectomycorrhizae. Eleven pine species grown in flats of soil infested with either of two isolates of *Thelephora terrestris* formed morphologically indistinguishable ectomycorrhizae. Noninoculated seedlings of the same pine species did not form ectomycorrhizae but developed dichotomously branched short roots and root hairs.

Outside the room, 90% of loblolly pine seedlings grown in pots developed four different ectomycorrhizal forms, regardless of treatment. The 11 species of pine in flats of initially uninfested soil formed abundant ectomycorrhizae with at least two airborne symbionts, one of which was *T. terrestris*.

Large numbers of pine seedlings with and without specific ectomycorrhizal associations can be studied in other than aseptic culture in the room. In this manner, natural forest conditions can be more closely duplicated for studies on ectomycorrhizal versus nonmycorrhizal relationships.

Introduction

Numerous problems have been encountered in investigations of root diseases and ectomycorrhizal[2] associations of forest trees under simulated natural conditions. The greatest problem has been undesirable fungal and bacterial contaminants which interact with the desired biological material. These interactions, either stimulatory or inhibitory, often nullify otherwise significant results (1). The presence of symbiont contaminants, presumably introduced as airborne basidiospores, has caused numerous failures in controlled experimentation on ectomycorrhizae in the field and greenhouse. Most significant works on ectomycorrhizal versus nonmycorrhizal relationships have been restricted to the unnatural condition of aseptic culture (2, 5, 11, 13).

In the southeastern United States, a heterogeneous mixture of ectomycorrhizal fungi exists in the soil of pine forests (14, 15). We have found that at least five culturally distinct symbionts of shortleaf pine seedlings colonize fumigated soil in the greenhouse within a few months, apparently from airborne spores (7). One of these symbionts is *Thelephora terrestris* (Ehrh.) Fr. These reports demonstrate the complexities of pine ectomycorrhizal associations and explain why one cannot grow noninoculated pine seedlings free of ectomycorrhizae in field or greenhouse culture.

Many problems can only be answered by the development of an artificial environment free of undesired ectomycorrhizal fungi. Some of these problems are the competition between ectomycorrhizal fungi and other soil organisms, the influence of ectomycorrhizae on the development of root disease, methods of inoculating soil with ectomycorrhizal fungi, the persistence of basidiospore and mycelial inoculum of various ectomycorrhizal fungi, seasonal and diurnal populations of spores of ectomycorrhizal fungi in various forested and nonforested sites, and methods of inducing basidiocarp production by ectomycorrhizal fungi.

The objectives of this research were (*a*) to design and construct a plant-growth room free of airborne ectomycorrhizal fungi and (*b*) to present results of initial studies in which several different pine hosts were grown in the room in soil infested with pure cultures of fungal symbionts.

Materials and Methods

Design, Construction, and Fumigation of Plant-growth Room

The room, 7.3 m × 3.4 m × 2.1 m, was constructed inside a greenhouse (Fig. 1). Two preentry rooms, each about 1.5 m × 1.7 m, were built at one end. The main growth room, about 5.5 m × 3.4 m, enclosed two green-

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[2]Terminology suggested by Peyronel *et al.* (10).

house benches. The framework was constructed of 5-cm × 10-cm studs and the ceiling joists were spaced 0.9 m apart. The roof was pitched to allow wash water to run off. The studs and joists were covered inside and outside with polyethylene plastic. The inside plastic (6 mil) was attached at all studs and joists with metal staples and polyethylene tape, and all joints were sealed with RTV-102 (General Electric Corp. Silicone Products Division, Waterford, N.Y.). The outside plastic (10 mil) was fastened only at the edges of the sheets to eliminate ripping which might result from plastic shrinkage. The lower 0.9 m of the sides of the room were covered with 0.64-cm tempered masonite. All masonite–plastic–wood joints were sealed with RTV-102 and nailed. The floor joint was sealed with an asphalt caulking compound. Aluminum doors were installed for each room. Wall fans (25 cm), controlled by two-way wall switches at each door, were installed for air removal and transfer.

The room was continuously air-conditioned by a unit with a cooling capacity of 37 000 B.T.U. Two 5000-W floor heaters maintained the air at temperatures above 15 °C. The supply and return air-duct systems were positioned under the greenhouse benches. Air volume and direction were controlled by dampers and registers. Two air-return vents, placed 45 cm from the ceiling at the end of each bench, were necessary to ensure equal air movement over the bench surfaces and to eliminate temperature stratification. An outside vent in the air-duct system continuously mixed about 5% outside air with the volume of air in the room. Two electronic air cleaners sized to remove particles larger than 0.03 to 0.1 μ were installed in the supply air duct.

Frequent entry into the growth room for plant watering was eliminated by the installation of an automatic pot-watering system with a regulator valve controlled by a time clock. A thermistor-based telethermometer system

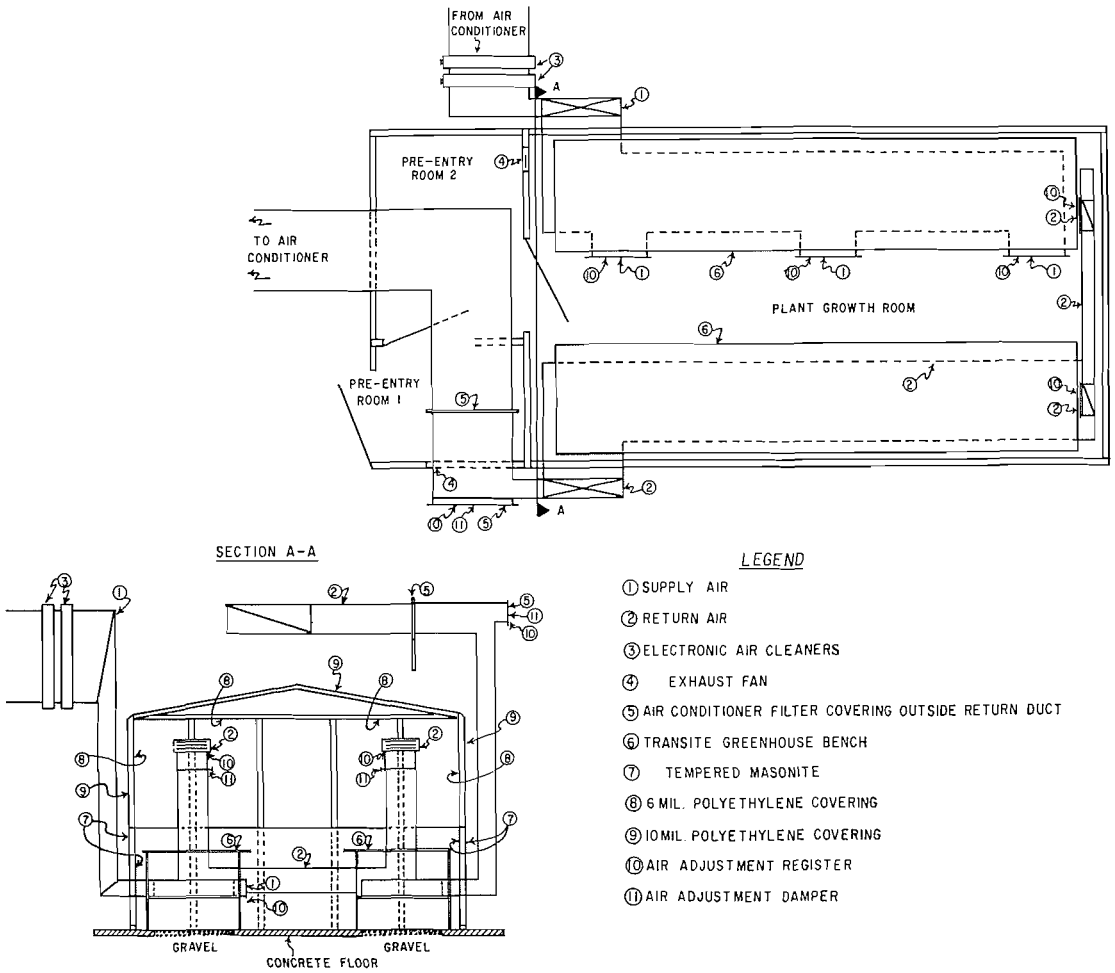

FIG. 1. Schematic diagram of electronically air-filtered, air-conditioned, plant-growth room.

with remote control outside the room sensed and recorded air and soil temperature several times daily from 11 locations in the room.

The gravel under the greenhouse benches was sterilized in place with a 1% mercuric chloride solution applied at the rate of 1 liter per 0.1 square meter of gravel surface. After all outside openings had been sealed and the inside return ducts had been closed, the rooms and their contents were fumigated with 9 lb (4.09 kg) of methyl bromide – chloropicrin mixture. The fumigant was injected at 60–65 °C at ceiling level through three ports in the masonite siding and circulated with two floor fans. Air temperature during fumigation exceeded 46 °C, and relative humidity was 80 to 90%. After 48 h, the outer door was partially opened, the air-conditioner, electronic cleaners, and wall fans were turned on, and the fumigant was exhausted and replaced with filtered air through the outside duct for return air. The fumigant was removed from the greenhouse by a large wall fan directly in front of the outer door of the growth room. After another 48 h, when all fumigant had been removed, the inside return ducts were opened and the outside return ducts were closed. Before entering the growth room, all personnel stopped in preentry room 2, washed their hands thoroughly in 60% ethyl alcohol, and donned sterilized coveralls with foot enclosures and surgical caps. All inoculum jars, pots, containers, flats, labels, vermiculite, perlite, etc. were either fumigated in the room, autoclaved, or washed in 60% ethyl alcohol.

The effectiveness of the fumigation treatment was tested by exposing plates of malt extract agar (Difco) on the bench surfaces and the floor for 30 min. Additional plates were taped over the supply and return air vents in a position facing the air flow. A series of plates was handled without being exposed to the air so that the number of contaminants from handling could be determined. For comparative purposes, plates were also exposed in the greenhouse outside the growth room. These plates were placed on benches and in front of the wall fan. Each of the 13 treatments was replicated 10 times. After exposure, the plates were incubated for 10 days at 25 °C and then examined for fungal and bacterial colonies.

Studies of Various Pine Species Inoculated and Noninoculated with Ectomycorrhizal Fungi

Eight inoculation techniques were tested on loblolly pine seedlings in new 10-cm clay pots. TREATMENT 1: Nonsterilized, finely divided humus from the A_o soil horizon of a loblolly pine forest was combined with a triple-autoclaved soil mixture at a ratio of 1:10. The soil mixture, which consisted of washed sand, peat moss, and loam soil in a 1:1:1 ratio by volume, was held at 20% water-holding capacity in 2-qt jars and autoclaved 3 times for 3-h periods. The autoclavings were separated by 3-day intervals. TREATMENT 2: Identical with treatment 1 except that the humus was triple-autoclaved. TREATMENT 3: Nonsterilized humus was combined with the triple-autoclaved soil mixture at a ratio of 1:2. TREATMENT 4: Identical with treatment 3 except that the humus was triple-autoclaved. TREATMENT 5: Inoculum of isolate 29 of *Pisolithus tinctorius* (Pers.) Coker & Couch, a symbiont of loblolly pine (8), was combined with the triple-autoclaved soil mixture at a ratio of 1:4. The inoculum was a 10-month-old culture which had been grown at 25 °C in 1 liter of vermiculite – peat moss substrate maintained at pH 5.5 (8) and moistened with 500 ml of modified Melin-Norkrans (MMN) liquid medium (6). TREATMENT 6: Identical with treatment 5 except that the inoculum of *P. tinctorius* was triple-autoclaved. TREATMENT 7: Control medium of noninfested vermiculite – peat moss – MMN was combined with the triple-autoclaved soil mixture. TREATMENT 8: The triple-autoclaved soil mixture alone.

Each of the two benches inside the growth room was divided into three 1-m areas (one on either end and one centered), and three pots from each treatment were placed at each of the six bench positions. Two benches in the greenhouse outside and on each side of the growth room were similarly divided, and three pots from each treatment were placed at each of these six bench positions. The groupings at each bench position were completely randomized.

The pots were watered thoroughly, planted with loblolly pine seed which had been surface sterilized (8), and covered with a 0.8-cm layer of sterile perlite so that light would be reflected from the soil surfaces. Fifteen-cm saucers were put under each pot, and a feeder line from the automatic watering system was placed in each. All pots received 65 to 70 ml of tap water (15 °C) at noon on alternate days for 30 days, after which they were automatically watered daily. The pots positioned on the benches in the greenhouse were similarly watered by hand. After 2 months, 30 ml of sterile Melin-Norkrans nutrient salt solution (6) were added to each pot with a sterile syringe.

Another study was installed in redwood flats. Inocula of isolates 2 (8) and Z-13 of *Thelephora terrestris* were prepared in the same manner as the inoculum of *P. tinctorius* used in the pot study. (Isolate Z-13 was furnished by B. Zak, Forestry Sciences Laboratory, Corvallis, Oregon.) The inoculum was combined with triple-autoclaved soil mixture at a ratio of 1:4 (v/v) and placed in flats. Seeds of the following pine species were surface sterilized in 30% H_2O_2 for 20 min: loblolly (*Pinus taeda* L.), shortleaf (*P. echinata* Mill.), longleaf (*P. palustris* Mill.), slash (*P. elliottii* Engelm. var. *elliottii*), Virginia (*P. virginiana* Mill.), sand (*P. clausa* (Chapm.) Vasey), Scots (*P. sylvestris* L.), sugar (*P. lambertiana* Dougl.), western white (*P. monticola* Dougl.), eastern white (*P. strobus* L.), and ponderosa (*P. ponderosa* Laws.). A 0.5-m row of each species was then planted in each flat and covered with 0.8 cm of sterile perlite. One flat was prepared for each isolate of *T. terrestris*, and two control flats were prepared from control medium as in treatment 7 of the pot study. The two *T. terrestris* flats and one control flat remained in the growth room, and the other control flat was placed outside the growth room on a bench in the greenhouse. Each flat was automatically watered with 520 to 560 ml of tap water (15 °C) at noon on alternate days for 30 days, then daily thereafter. The control flat in the greenhouse was similarly watered. After 2 months, 250 ml of sterile Melin-Norkrans nutrient salts (6) was added to each flat.

After 5 months the studies were terminated and the seedlings were examined for relative vigor and feeder-root development. From each treatment, 30 ectomycorrhizae or nonmycorrhizal short roots were either macerated and examined microscopically in phloxine and lactophenol (control seedlings grown inside room only) or fixed, dehydrated, embedded, serially sectioned (8 μ), and stained in safranine–fast green (8) for more detailed histological examination.

Results

Design, Construction, and Fumigation of Plant-growth Room

A positive air pressure in the room was essential for maintenance of a spore-free environment. A constant air pressure of 0.5 mbar was achieved by partially closing return-duct dampers. This pressure was transferred to pre-entry rooms by wall fans which were turned on when doors were opened.

Examination of the exposed petri plates which had been placed in the growth room to check on its sterility after fumigation showed an average of 0.15 fungal and 0.21 bacterial colonies per plate. Other plates that had been similarly handled but not exposed developed even more colonies, demonstrating that the fumigation process had, in fact, eliminated fungal and bacterial populations. The fungal contaminant picked up inside the growth room, a species of *Penicillium*, had undoubtedly been carried in on the surfaces of the petri plates. The number of contaminants in the greenhouse averaged 29.8 fungal and 5.8 bacterial colonies per plate. At least seven different genera of fungi and several different bacterial colony types were detected on plates exposed in the greenhouse. The few bacteria detected inside the growth room were culturally identical with those detected on handled but nonexposed plates.

Measurements of light quantity at various times of the day indicated that from 43 to 58 klux of light penetrated the double plastic covering enclosing the growth room, about 13 klux less than that measured in the greenhouse during the same periods.

Soil temperatures in pots and flats during daylight hours inside the growth room averaged 18.1 °C (range 16.8 to 19.5 °C) as compared to soil temperatures in the greenhouse which averaged 21.5 °C (range 19.4 to 29.0 °C). Air temperatures at ceiling height inside the growth room averaged 25.3 °C (range 24.8 to 25.7 °C), and air temperatures at bench height averaged 25.2 °C (range 23.7 to 26.5 °C). Night temperatures in the room averaged 18 °C (range 15 to 19 °C). Air temperatures in the greenhouse fluctuated between 23 and 32 °C during daylight hours and ranged from 19 to 24 °C at night. Relative humidity inside the room varied between 32 and 54%.

Studies of Various Pine Species Inoculated and Noninoculated with Ectomycorrhizal Fungi

Overall survival of the potted loblolly pine seedlings was 90% inside the growth room and 67% in the greenhouse. Survival inside the growth room at the different bench positions was 85, 87, 93, 96, 94, and 85%, whereas survival at corresponding bench positions outside the growth room was 17, 21, 83, 83, 91, and 91%, respectively. The low survival at the first two bench positions outside the room suggests that bench position exerted a strong influence on the survival of seedlings in the greenhouse. The relative vigor of all loblolly pine seedlings inside the growth room was similar, regardless of treatment or bench position. All these seedlings exhibited dark green foliage and ranged from 10 to 12 cm in height. Since bench position did not cause a significant difference in seedling survival or growth inside the room, the following results pertain to combined data from all bench positions.

Monopodial and bifurcate ectomycorrhizae with well-defined fungus mantles and Hartig nets were observed on all loblolly seedlings which had been grown in the nonsterile humus and soil mixture combined at the ratio of 1:10. Ectomycorrhizae were not detected on seedlings which had been grown in the nonsterile humus and soil mixture (1:2 ratios) nor on those grown in viable inoculum of *Pisolithus tinctorius*. Seedlings from the remaining treatments involving sterile humus, sterile inoculum of *P. tinctorius*, control nutrient substrate, or soil mixture were free of ectomycorrhizae. All seedlings without ectomycorrhizae had numerous root hairs and bifurcate short roots. *Pisolithus tinctorius*, a symbiont with a high temperature optimum (8), probably failed to form ectomycorrhizae because of the low soil temperatures maintained in the growth room.

The results from root examinations of loblolly pine seedlings grown in pots outside the

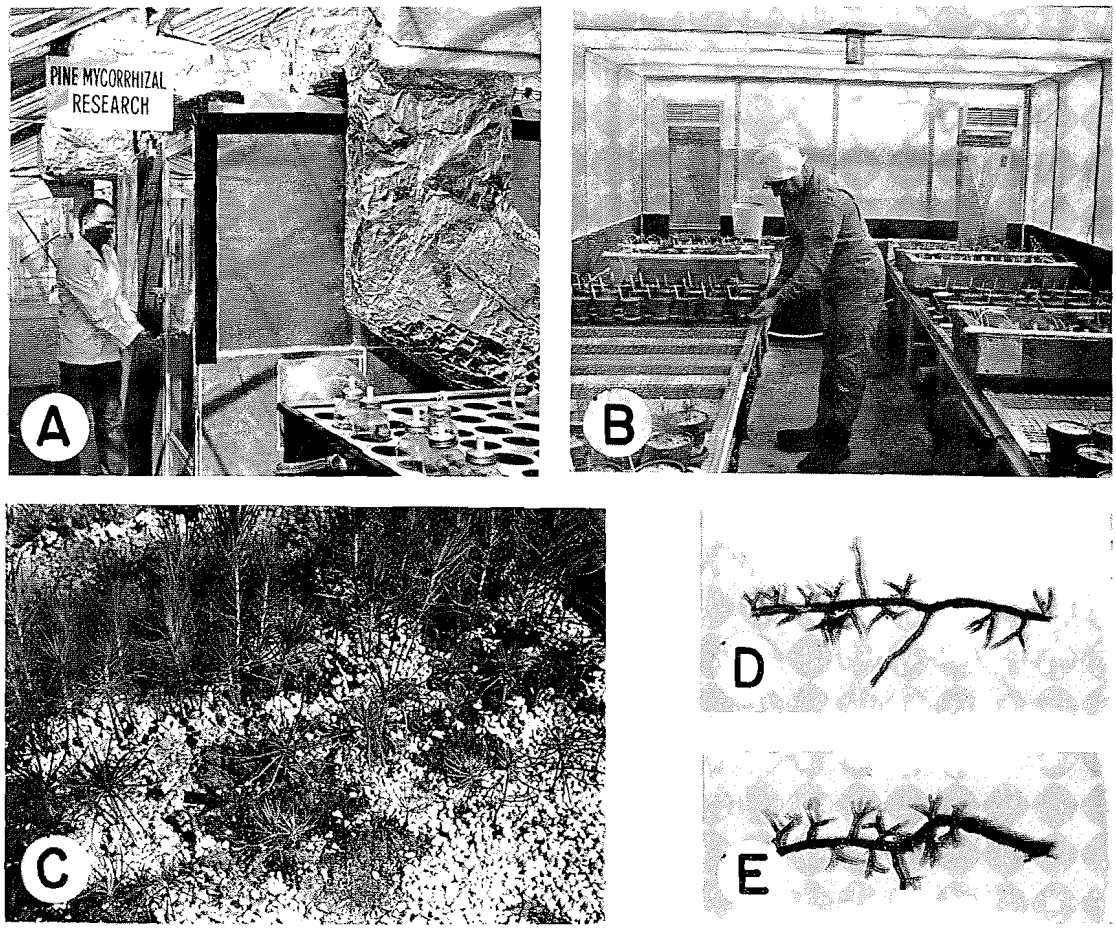

FIG. 2. Initial studies in plant-growth room. Greenhouse view (A), inside view (B), sporophores of *Thelephora terrestris* isolate 2 formed after 4 months in seedling flats (C), ectomycorrhizae from slash pine seedlings formed by *T. terrestris* isolate 2 (D), and ectomycorrhizae from slash pine seedlings formed by isolate Z-13 (E).

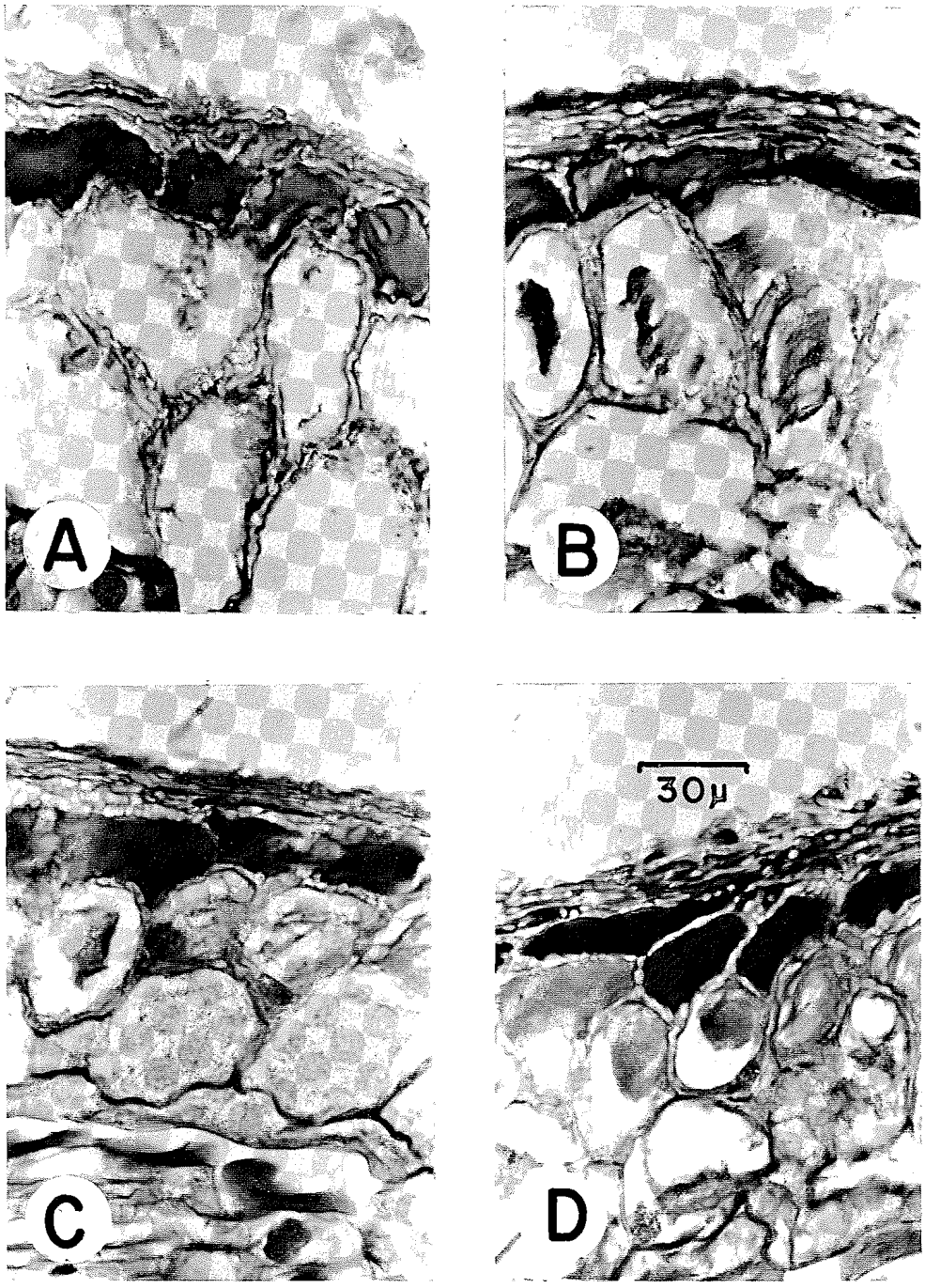

FIG. 3. Longitudinal sections of ectomycorrhizae formed in the plant-growth room on slash pine (A and B) and eastern white pine (C and D) by *Thelephora terrestris* isolate 2 (A and C) and isolate Z-13 (B and D).

growth room were strikingly different from those of seedlings grown in pots inside the growth room. Over 90% of all seedlings in the greenhouse formed ectomycorrhizae, regardless of soil treatment or bench position. The degree of ectomycorrhizal development varied considerably within and between each treatment. However, at least four different morphological forms of ectomycorrhizae were represented. Very few root hairs were observed on nonmycorrhizal short roots or on lateral root tips of those seedlings. The roots were less succulent than those of seedlings grown in the growth room.

One week before termination of the flat study, both introduced isolates of *T. terrestris* produced basidiocarps on the perlite surface and on seedling stems of all pine species. Both isolates formed ectomycorrhizae on roots of all 11 pine species (Fig. 2). Mycelial strands of *T. terrestris* were abundant in the root zones and were readily traced from basidiocarps to the ectomycorrhizae. All ectomycorrhizae were morphologically indistinguishable, although slight variations in form and in thickness of fungus mantle were observed (Table I). Most of the ectomycorrhizae for each association were white to cream and bifurcate to coralloid. All ectomycorrhizae had well-formed fungus mantles and Hartig net development to the endodermis or to within one cortex cell layer of the endodermis (Fig. 3). *T. terrestris* isolate 2 was reisolated on MMN agar medium from ectomycorrhizae of slash and eastern white pines; mercuric chloride at 100 p.p.m. for 2½ min was used as the surface sterilant (9). Isolate Z-13 was not reisolated from any of the 20 ectomycorrhizae plated per pine species, perhaps because its growth rate is slower than that of isolate 2 (7).

Seedlings of the 11 pine species from the control flat inside the growth room were free of ectomycorrhizae. All feeder roots were extremely succulent and had abundant root hairs. Many

TABLE I

Macroscopic and microscopic characteristics of ectomycorrhizae formed on roots of 11 pine species by *Thelephora terrestris* in the plant-growth room

Pine species	*Thelephora terrestris* isolate 2			*Thelephora terrestris* isolate Z-13		
	Rated ectomycorrhizal development[a]	Mantle thickness, μ		Rated ectomycorrhizal development	Mantle thickness, μ	
		Av.[b]	Range		Av.	Range
Pinus echinata shortleaf	Excellent	17.5	10–25	Excellent	14.8	9–25
P. taeda loblolly	Excellent	16.0	10–25	Excellent	15.5	10–25
P. elliottii var. *elliottii* slash	Excellent	13.5	10–20	Excellent	19.0	10–30
P. palustris longleaf	Excellent	14.6	10–18	Moderate	16.2	10–23
P. strobus eastern white	Excellent	12.8	9–25	Excellent	16.5	10–25
P. monticola western white	Good	15.3	10–23	Moderate	13.8	10–21
P. lambertiana sugar	Excellent	12.6	9–26	Good	13.2	9–22
P. virginiana Virginia	Excellent	12.5	8–18	Excellent	18.2	10–32
P. sylvestris Scots	Moderate	14.0	10–26	Excellent	18.4	10–31
P. ponderosa ponderosa	Moderate	14.2	5–22	Excellent	13.0	10–25
P. clausa sand	Poor	14.0	6–20	Moderate	19.5	12–30

[a]Rating based on macroscopic examination of 50–70 seedlings of each species. Excellent, about 75–100% of feeder roots ectomycorrhizal; good, 50–74%; moderate, 25–49%; poor, 1–24%.
[b]Average of 50–85 ectomycorrhizal fungus mantles measured at 400× from histological slides.

pine species formed nonmycorrhizal short roots which were dichotomously branched. No sporophores of any fungus were observed in this control flat. In contrast, seedlings of all 11 pine species from the control flat maintained outside the growth room developed abundant ectomycorrhizae, and *T. terrestris* and an unidentified discomycete fruited on the perlite surface. Macroscopically, the ectomycorrhizae, which were similar for all pine species, were white to cream in color and monopodial, bifurcate, or coralloid. Histologically, two fungal symbionts were readily distinguished; for simplicity, they were designated type 1 and type 2. The ectomycorrhizae of type 1 were identical with those formed by either of the two isolates of *T. terrestris* in the flats of inoculated seedlings. The fungal symbiont of ectomycorrhizal type 2 had very large hyphal diameters which averaged 5.8 μ and ranged from 5.4 to 6.8 μ. This fungus formed few septa and no clamp connections. Attempts at reisolating these two fungal symbionts from over 300 ectomycorrhizae were unsuccessful.

Discussion

The results of these studies indicate that the plant-growth room is a complete success. Noninoculated pine seedlings did not form ectomycorrhizae. The quantity of light and the temperature of the air and soil provided excellent growing conditions for pine and for development of ectomycorrhizae from inoculations. Seedlings inside the room were more vigorous and healthy than seedlings grown outside the growth room. The major factors contributing to the success of the room are the highly effective electronic air cleaners, the constant positive air pressure which can be maintained in the room, and the sterile outer clothing which is worn to minimize spore entry on one's person. In addition, construction of the plant-growth room inside a greenhouse eliminated direct exposure of the plastic to the elements. A minimum of maintenance entries is required because of the automatic watering system and the equipment for remote sensing of temperatures. The cost of the entire room and components is about that of a small, self-contained growth chamber. Despite this relative low cost, the growth room has over 18 square meters of plant-growing space with excellent environmental control and can be entered for installation, maintenance, and dismantling of studies. The major advantage, however, is that microbial contaminants are effectively controlled by fumigation and electronic air-filtration. Pine seedlings can therefore be grown free of ectomycorrhizal fungi introduced from airborne spores. These results show that it is now possible to grow large numbers of pine seedlings with and without specific ectomycorrhizae in other than aseptic culture. By using this capability, we can more closely duplicate natural forest conditions for experimentation on ectomycorrhizal and nonmycorrhizal relationships.

Before this report, the known host range of *Thelephora terrestris* was limited to slash (15), Virginia (4), eastern white (3), pitch (*Pinus rigida* Mill.) (12), and shortleaf (9) pines. The host range should now be expanded to include loblolly, longleaf, western white, sugar, Scots, ponderosa, and sand pines. This is the first report of pure-culture synthesis of ectomycorrhizae on roots of sand pine.

The development of basidiocarps by both isolates of *T. terrestris* confirms the identity of these isolates. This point has been questionable since each isolate in culture is completely vegetative (9) and each was obtained directly from ectomycorrhizae and not from a basidiocarp. The fructification of *T. terrestris* in the growth room is indicative of favorable growing conditions for other hymenomycetes in subsequent research. All ectomycorrhizae formed by both isolates of *T. terrestris* were indistinguishable on the pines tested. This fact suggests that the symbiont exerts the controlling influence on both the fungus mantle color and the morphological configuration of ectomycorrhizae.

Acknowledgment

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